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Epidermal Hyperplasia in Mouse Skin Following Treatment with Alternative Drinking Water Disinfectants

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Female SENCAR mice were treated with aqueous solutions of hypochlorous acid (HOCl), sodium hypochlorite (NaOCl), chlorine dioxide (ClO_2), and monochloramine (NH_2Cl) by whole body exposure (except head) for a 10-min period for 4 days in the first experiment and for 1 day (except NH_2Cl) in the second experiment. Animals were sacrificed the day following the last treatment (experiment 1) or on day 1, 2, 3, 4, 5, 8, 10, and 12 following treatment (experiment 2), and skin thickness was measured by light microscopy at ×400 by use of an eyepiece micrometer. Concentrations of disinfectants were 1, 10, 100, 300, and 1000 mg/L, for experiment 1 and 1000 mg/L for experiment 2. Thickness of the interfollicular epidermis (IFE) for control animals was 15.4 ± 1.5 μm. After 4 days of treatment at 1000 mg/L, HOCl and ClO_2 increased thickness to 39 ± 7.0 and 40.2 ± 11.8, and NaOCl increased thickness to 25.2 ± 6.1 μm. Only HOCl and ClO_2 were tested at 300 mg/L, yielding an IFE thickness of 30.0 ± 13.1 and 16.8 ± 0.8 μm, respectively. The response to HOCl was found to be dose-related; the minimally effective dose was 100 mg/L. In earlier, preliminary tests to determine optimum treatment schedule, the response to HOCl appeared to be maximal after 4 days of treatment and tended to decrease with further treatment. The time-course study following a single treatment of 1000 mg/L HOCl, however, showed a progression of IFE thickening of from 18.3 ± 1.4 at 1 day to 30.8 ± 8.0 at 8 days, decreasing to 19.1 ± 6.2 μm at 12 days. ClO_2 and NaOCl when tested in this manner did not produce increased thickness of IFE with time, but rather gave a persistent level of increase that remained for the 12 days. NH_2Cl reduced skin thickness to 13.6 ± 6.1 μm. Examination of sections of skin treated with HOCl and ClO_2 indicated an increase in cell numbers. HOCl and ClO_2 are therefore capable of inducing hyperplastic responses in the mouse skin. The basis for the decrease in skin thickness resulting from NH_2Cl treatment remains to be established.

Introduction

Alternative methods for disinfecting drinking water supplies are still being considered by the U.S. Environmental Protection Agency (EPA) as a means to minimize the formation of halogenated by-products associated with chlorination. In the past decade it has become increasingly apparent that a number of potential health hazards are associated with disinfection of potable water (*1*). In general, the hazards associated with ingestion of disinfectants or their by-products have received the most attention. Dermal exposure to drinking water disinfectants is, however, almost universal, and the toxicology associated with this route must also be characterized.

In the present studies, hypochlorous acid (HOCl), sodium hypochlorite (NaOCl), chlorine dioxide (ClO_2), and monochloramine (NH_2Cl) were tested for hyperplasiogenic activity in SENCAR mice. These studies follow from previous observations that chlorine is capable of producing hyperplasia (*2*) and that it can act as a cocarcinogen in mouse skin (*3*). Our ultimate purpose is to clarify the extent to which alternative forms of drinking water disinfection may be capable of promoting the growth of tumors in the skin. The present results are concerned exclusively with the relative degree of proliferative epidermal hyperplasia produced by these chemicals.

Methods

Chemicals

Sodium hypochlorite (NaOCl) was prepared by bubbling chlorine gas into a 40 g/L sodium hydroxide (NaOH) solution until a pH of 8.5 was achieved. To obtain the hypochlorite (OCl^-) solution, a portion of NaOCl solution taken to pH 12.0 by continued chlorine

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bubbling was titrated to a pH of 6.5 with 2.5 N hydrochloric acid (HCl). NH_2Cl solutions were prepared by titrating a 1.75% ammonium hydroxide (NH_4OH) solution with a NaOCl (31 g/L chlorine [Cl]) solution until a 1.4% NH_4OH solution was achieved. The resulting NH_2Cl solution was adjusted to the desired concentration with distilled water, and pH was adjusted to the desired 8.5 to 8.7 with 2.5 N HCl. ClO_2 was prepared by purging from an acidified-$NaClO_2$ generator, through an absorbent $NaClO_2$ column, into distilled deionized water to the desired concentration. 12-O-Tetradecanoylphorbol-13-acetate (TPA) was obtained from Chemical Carcinogenesis Company (Eden Prairie, MN).

Assay

Female SENCAR mice, 6 to 7 weeks of age, were used in these studies. Three days before beginning treatment, the backs of the animals were shaved. At the start of treatment, only those mice found to be in the resting phase of the hair cycle were used. Treatments with disinfectants or water were administered using a series of small Plexiglas chambers that were designed to hold the animals while the compartments were flooded with test solutions and to protect them from inhalation of the toxic vapors during the treatment procedure. A preliminary time-course study was conducted to determine the most effective treatment duration for yielding epidermal hyperplasia. In this study, five animals per group were exposed to 1000 ppm HOCl solution for 10 min per day for 1, 2, 3, 4, 5, 7, and 8 days. Based on the results of that study, animals in the primary study were treated for 4 days with 1, 10, 100, 300, or 1000 ppm of HOCl, ClO_2, or NH_2Cl, with the exception that NH_2Cl was not tested at 300 ppm. In addition, NaOCl was tested at 1000 ppm for 4 days of treatment. Negative controls received distilled water for the same duration. All whole-body (except head) exposures consisted of a 10-min contact time per day of treatment. TPA was applied topically at a dose of 1.0 μg in 0.2 mL acetone per mouse and served as the positive control. In a separate study, a single treatment of HOCl, NaOCl, or ClO_2 was administered in the same manner, and animals were held for up to 12 days before sacrifice to determine the time course associated with the hyperplastic response. This study was conducted in two phases, each phase containing a separate distilled water group as a negative control (phase 1, HOCl and ClO_2; phase 2, NaOCl and TPA). Animals in each study were sacrificed 24 hr after final treatment, and a 1-cm^2 section of dorsal skin was taken from each mouse for evaluation. These sections were immediately immersed in Millonig's phosphate buffer, pH 7.3, at 4°C for 5 sec, then transferred to a drop of Karnovsky's fixative at 4°C and minced into 1-mm^2 pieces. After 24 hr of further fixation in Karnovsky's fixative at 4°C, these sections were postfixed in 2% buffered osmium tetroxide, dehydrated in ethanol, and embedded in Spurr epoxy resin (Electron Microscopy Sciences, Fort Washington, PA) with the skin oriented to allow perpendicular cuts. Six

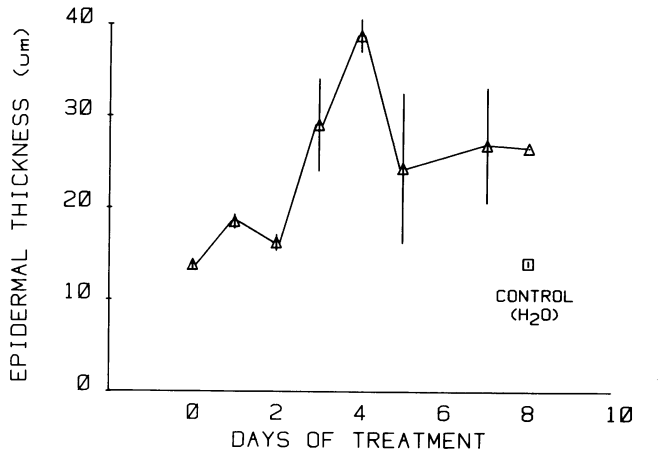


FIGURE 1. Effect of repetitive treatments of 1000 ppm HOCl on epidermal thickness. Animals were sacrificed 24 hr following final treatment. Zero (0) days represents nontreated animals that were sacrificed on day 1. Water control animals received 8 days of treatment and were sacrificed on day 9. The thickness of the IFE was measured with an eyepiece micrometer at ×400. Values represent the group mean ± SE. $N = 5$.

nonserial 1.0-μm sections from each block were cut, stained with 1% toluidine blue, and examined with a light microscope. Epidermal thickness and the cell counts were measured at ×400 with an eyepiece micrometer.

Results

Figure 1 depicts the effect of multiple treatments of 1000 ppm HOCl on epidermal thickness measured 1 day after the last treatment. From these data we concluded that four repeated daily 10-min applications of HOCl provided a maximal response. This period of exposure substantially depletes the HOCl concentration (i.e., > 70%); consequently, using longer exposure periods in this static system was pointless. The maximal epidermal thickness observed was 38.7 μm (± 4.0 SE) compared to 13.8 μm (± 1.1 SE) for animals exposed to distilled water treatments.

The hyperplasiogenic activity of the four alternative disinfectant solutions tested in the primary study is summarized in Table 1. The epidermal layer in control animals measured 15.4 μm. When four daily treatments of 1, 10, 100, 300, and 1000 ppm HOCl were applied and animals were sacrificed on day 5, results for 1 and 10 ppm were not unlike those of controls (14.4 and 15.8 μm), but 100, 300, and 1000 ppm progressively increased skin thickness to 21.9, 30.0, and 38.7 μm (significant at $p < 0.05$), respectively. In contrast, when animals were exposed for 4 days to OCl^- (pH 8.5) at a concentration of 1000 ppm and sacrificed on day 5, the epidermal thickness was increased to only 25.0 μm. The results observed with ClO_2 treatment were similar to those found with HOCl treatment. Treatments of 1, 10, 100, 300, 1000 ppm ClO_2 followed by sacrifice on day 5

Table 1. Skin hyperplasia produced by alternate drinking water disinfectants—dose response.

Treatment	Dose ppm[a]	Days of treatment	Epidermal thickness, μm[b]	Cell count/mm	
				Total	Basal
Control (H_2O)		4	15.4 ± 1.5	38 ± 6.8	24 ± 3.5
Chlorine pH 6.5	1000	4	38.7[c] ± 7.0	62[d] ± 19.3	27 ± 4.1
	300	4	30.0 ± 13.0	77[d] ± 14.0	33[d] ± 7.0
	100	4	15.8 ± 2.5	40 ± 5.2	24 ± 5.0
	1	4	14.4 ± 1.7	37 ± 6.4	25 ± 4.7
Chlorine pH 8.5	1000	4	25.0[c] ± 6.2	61[d] ± 8.8	25 ± 5.7
Chlorine dioxide	1000	4	40.2[c] ± 11.8	70[d] ± 9.0	32[d] ± 6.2
	300	4	16.8 ± 0.8	77[d] ± 11.0	35[d] ± 5.0
	100	4	13.5 ± 2.2	37 ± 6.0	25 ± 3.2
	10	4	15.0 ± 1.2	37 ± 3.7	26 ± 4.4
	1	4	15.5 ± 2.5	38 ± 5.3	26 ± 2.6
Chloramine	1000	4	13.6 ± 6.1	35 ± 3.6	25 ± 2.8
	100	4	13.1 ± 2.3	35 ± 3.0	22 ± 2.1
	10	4	14.4 ± 1.1	37 ± 5.0	25 ± 4.1
	1	4	14.0 ± 1.6	36 ± 6.3	21 ± 2.0
TPA	1.0 μg	1	32.8 ± 3.4	43[d] ± 5.7	20 ± 3.5

[a] Whole body (except head) exposure to solutions consisted of 10 min contact time per day of treatment in plexiglass chambers designed to protect animals from toxic vapors. TPA was administered topically to the back of the animals. ($N = 5$).

[b] Thickness of IFE was measured at × 600 with light microscopic using an eyepiece micrometer. Values shown are mean per group ± standard deviation.

[c] Significant at $p < 0.05$. Statistical analyses were performed using Tukey's multiple comparison test.

[d] Significantly different from control at $p < 0.05$. Statistical analyses were performed using Tukey's multiple comparison test.

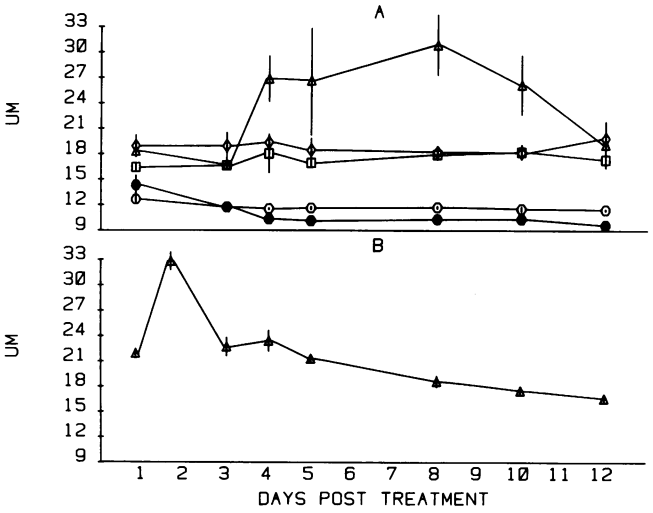
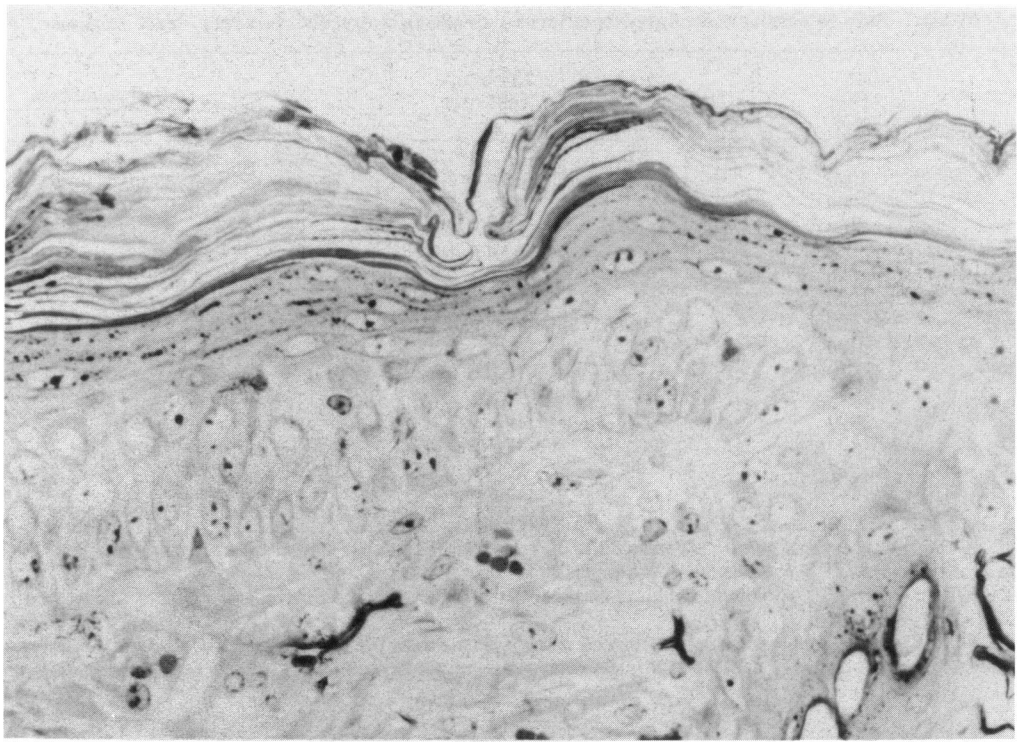


FIGURE 2. Time course of hyperplasiogenic response of the SENCAR mouse skin after a single treatment with (*A*) 1000 ppm HOCl (△), NaOCl (□), ClO_2 (◇), or distilled H_2O (○) control for HOCl and ClO_2 (●) control for NaOCl; (*B*) 2.5 μg 12-O-tetradecanoylphorbol-13-acetate (TPA). There were five animals per treatment group at each time of sacrifice. Animals were sacrificed and skin thickness measurements made at the indicated intervals following treatment. Measurements represent average thickness of IFE determined with an eyepiece micrometer at ×400. Values represent the group mean ± SE.

did not increase epidermal thickness at the four lower doses (15.5, 15.0, 16.8, and 13.5 μm), but did result in a significant response ($p < 0.05$) at the high dose (40.8 μm). NH_2Cl, at all dose levels, demonstrated a tendency to decrease epidermal thickness, although not significantly below controls. A single dose of TPA at 1.0 μg yielded a skin thickness of 32.8 ± 3.4 μm on the fifth experimental day.

Cell counts (Table 1) indicate that proliferative activity contributes to the hyperplasiogenic action of the disinfectant agents. To demonstrate this proliferation, the effect of the most active disinfectant doses (1000 ppm × 4) were compared with those of the vehicle control (H_2O). There were 62/27 (total/basal) cells per linear millimeter with HOCl, 61/25 with OCl^-, 70/32 with ClO_2, and 35/25 with NH_2Cl, compared to 38/24 cell/mm with distilled water treatments. TPA treatment resulted in 43/20 cells/mm.

It is obvious that the cell counts for OCl^- are not consistent with the reduced IFE thickness resulting from 1000 ppm OCl^- (Table 1). When compared to cell count/epidermal thickness relationship noted in groups receiving HOCl or ClO_2 as the same dose level. Cell counts for OCl^- (61/25-total/basal) were similar to counts found in HOCl and ClO_2 (62/27 and 70/32, respectively). The IFE thickness of OCl^- treated groups (25 ± 6.2 μm), however, is only 64% of the measurement seen with HOCl and ClO_2 (38.7 ± 7.0 and 40.2 ± 11.8 μm, respectively). This discrepancy is explained in part by the presence of an increased number of small cells in the stratum spinosum of skin sections from the OCl^- group compared to sections from HOCl- and ClO_2-treated animals. Various combinations of changes in the basal layer such as widening and narrowing of elongation of cells and the degree of intracellular space may account for the differences noted in the basal cell counts. The low basal cell count noted in the TPA 1.0 μg group (20 ± 3.5 μm) could possibly be explained by the presence of elongated, wide basal cells with prominent gaps



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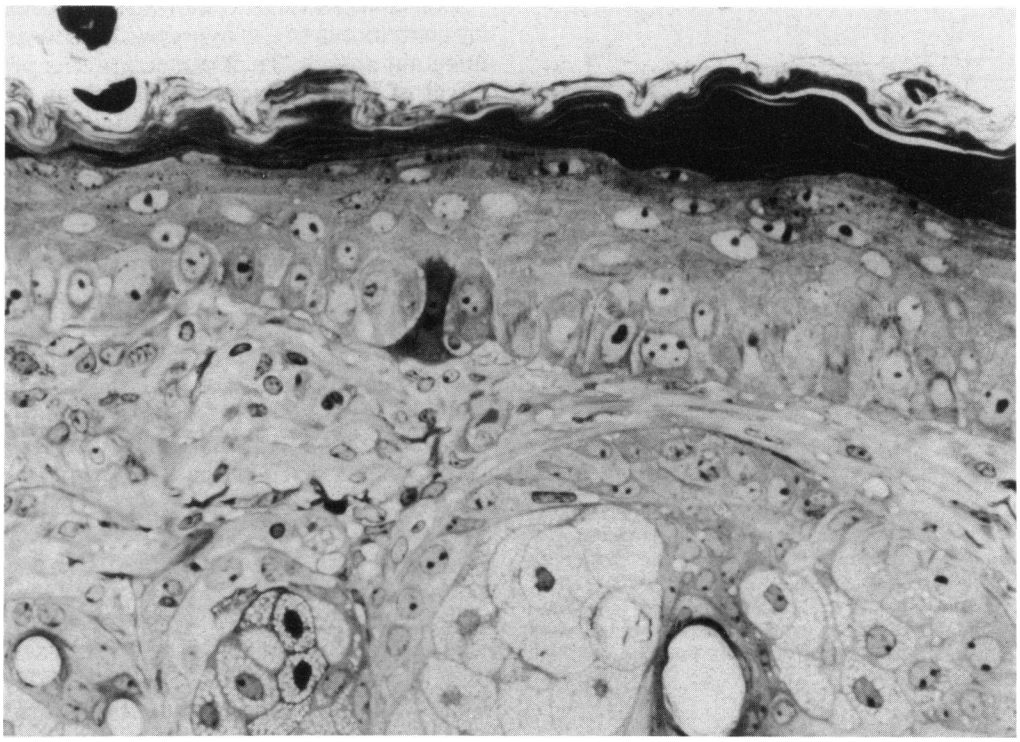

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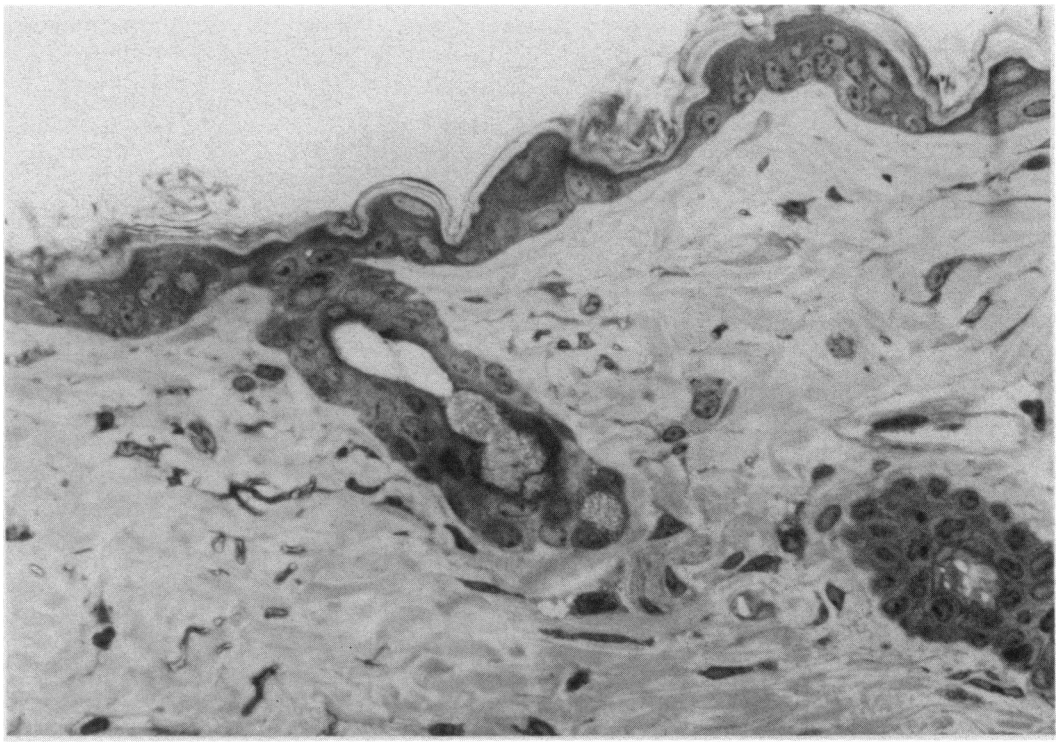

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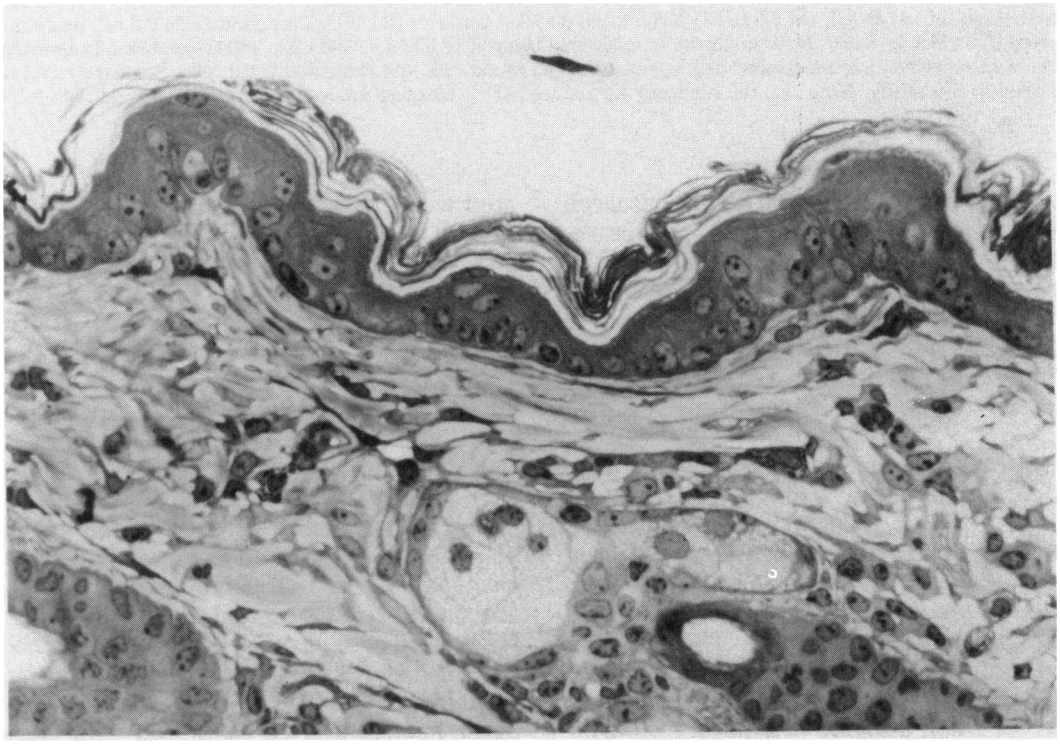

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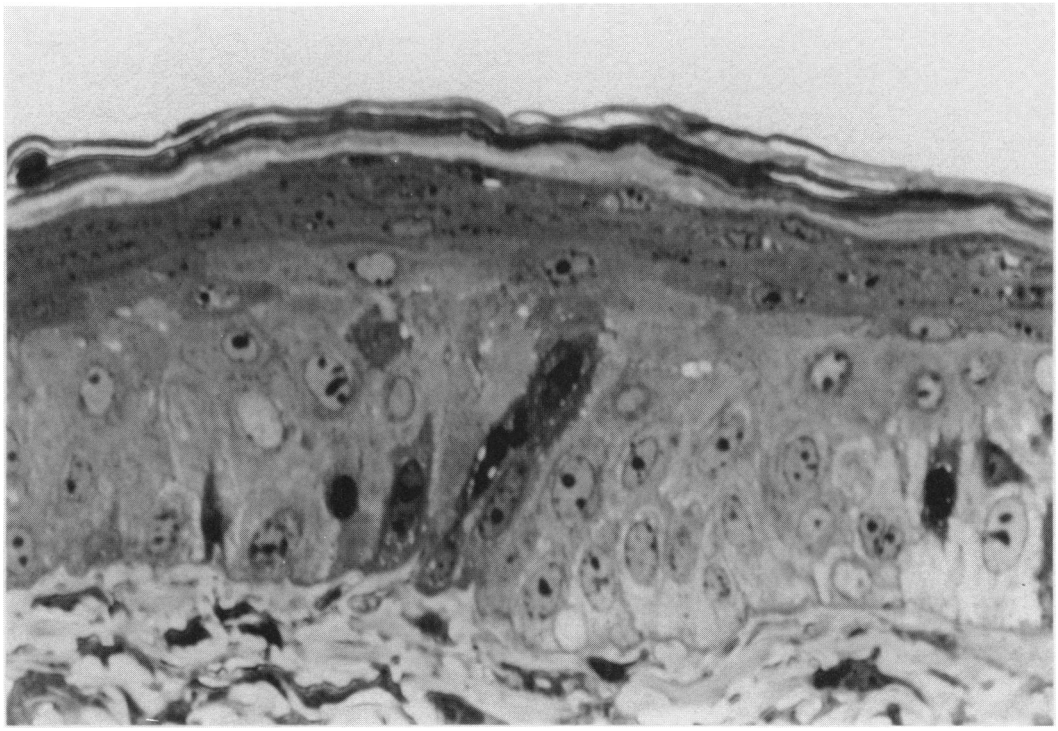

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FIGURE 3. Micrographs depicting thickness of IFE of SENCAR mouse skin after four daily 10-min whole body (except head) treatments with 1000 ppm solutions of (*A*) HOCl, (*B*) ClO_2, (*C*) NH_2Cl, (*D*) distilled water or (*E*) TPA 2.5 μg/mouse in 0.2 mL acetone applied to back only. ×600 except (*E*) which is ×950. Note similarity of epidermal layer of $HOCl^-$ (*A*) and ClO_2^-(*B*) treated skin to that treated with TPA (*E*). Observable in these sections are increased cell layers, enlarged round cells and elongated basal cells. Some dark cells are present but were not quantified in this study. Note also the tendency for reduced IFE thickness associated with NH_2Cl treatment (*C*) as compared to control (*D*).

between cells. This is in contrast to the higher basal cell count observed in HOCl, OCl^-, and ClO_2 groups receiving 1000 ppm solutions (27 ± 4.1, 25 ± 5.7, and 32 ± 6.2 μm, respectively). Elongation of basal cells does not occur in animals treated with only water. Final explanation of the exact association between cell count and induced epidermal thickness may require more detailed study possibly using morphometric analysis procedures.

The hyperplastic response measured throughout a 12-day recovery period following a single treatment of HOCl, NaOCl, or ClO_2 at 1000 ppm is presented in Figure 2. There were five animals per treatment per day of evaluation. Again HOCl resulted in the greatest response elicited by the disinfectant agents (30.8 μm), nearly equaling that of TPA (32.8 μm). The response to HOCl, however, was delayed for 4 days whereas maximum response to TPA occurred on the second day. The hyperplasia resulting from HOCl exposure was sustained considerably longer than that associated with TPA treatment. The epidermal thickness after TPA treatment peaked at 32.8 μm on day 2 and dropped sharply to 22. 7 μm on day 3. Thereafter, it gradually decreased to 16.6 μm on day 12. The thickness associated with HOCl treatment peaked significantly on day 8 at 30.8 μm after a sharp increase to 26.8 μm on day 4 ($p < 0.05$) and was still at 26.1 μm on day 10 before falling to 19.1 μm on day 12. The increase with both NaOCl and ClO_2 was less than with HOCl, but was significant ($p < .05$) and was sustained throughout the 12-day period, with the highest values reached at day 10 (18.2 μm) and day 12 (19.8 μm), respectively. The phase one control values ranged from 11.2 μm (day 8) to 12.6 μm (day 1). The phase two control values ranged from 9.7 μm (day 12) to 14.3 μm (day 1).

The morphological features of the epidermal hyperplasia are demonstrated in Figure 3. The morphological changes observed after treatment with HOCl or ClO_2 are similar to those found 24 to 48 hr after TPA treatment (*4*). These changes include a thickened epidermis, 4 to 6 cell layers thick, consisting of enlarged round cells with prominent, intracellular bridges and hyperkeratosis. The basal cells, containing oval nuclei, are elongated and lie perpendicular to the basement membrane. These changes, comparable to those produced by TPA, are more evident in the ClO_2 group than in the HOCl group. The epidermis of mice treated with NH_2Cl is reduced in thickness but otherwise is not remarkably

different when compared to the epidermis of animals receiving distilled water.

Discussion

The present work demonstrated that brief exposures of mouse skin to relatively high concentrations of most of the drinking water disinfectants evaluated resulted in hyperplastic responses. The major exception to this observation was NH_2Cl, which failed to increase epidermal thickness or cell counts at concentrations of up to 1000 mg/L. ClO_2 and both forms of chlorine (HOCl and OCl^-) increased cell counts to an equivalent extent at 1000 mg/L, but the increase of the thickness of the epidermal layer observed with OCl^- was only 40% of that observed with HOCl and ClO_2. The sections used in these preliminary experiments are currently undergoing review to determine whether the differences in epidermal thickness reflect differences in cellular density or cell size in the OCl^--treated animals.

The maximal hyperplastic response to HOCl initially appeared to be dependent on multiple applications. However, subsequent experimentation made it clear that the response to a single application was delayed relative to the response to TPA. Consequently, it is not clear that four treatments are required for maximal response. In contrast, the application of a single treatment of OCl^- or ClO_2 produced an increase in epidermal thickness that could be observed within 24 hr and that was maintained for 12 days. Comparing the data following 1 day of teatment for these latter two compounds with those observed with four repeated treatments (Table 1) indicates that a maximal response requires either repeated or more prolonged exposures. The rapid depletion of active chlorine from HOCl, OCl^-, and ClO_2 (but not from NH_2Cl) does not allow differentiation between these two possibilities. In all cases, the role of concentration versus dose needs to be further explored experimentally.

Within the conditions of the present study, HOCl is clearly the more potent hyperplasiogenic compound studied. It increased epidermal thickness at levels as low as 300 mg/L with four daily, 10-min treatments. The extent to which this result might be extended to lower concentrations with more prolonged exposures, with some provision for maintaining a constant chlorine residual, remains to be determined. Somewhat surprisingly, repeated applications of HOCl at 1000 mg/L did not appear to be particularly effective in maintaining the maximal response. This lack of effectiveness may be attributed to either ineffective penetration of the reactive species through the hyperplastic skin (i.e., a type of adaptation) or toxicity to the surface layer of cells causing more rapid loss of these cells with subsequent treatments.

Pfeiffer (*2*) examined the effects of repeated topical applications of 1% NaOCl (approximately 0.2 mg free Cl per animal) and NH_2Cl solutions to the skin of NMRI mice. Neither OCl^- nor NH_2Cl appeared to influence the development of tumors significantly, whether applied before, with, or after applications of benzo(a)pyrene. Prior work by Hyatsu (*3*) had indicated that NaOCl (approximately 5 mg per mouse per dose) did enhance the development of tumors initiated by 4-nitroquinoline-1-oxide. More recent work by Kurokawa (*7*) involved the application of 0.2 mL of 1% NaOCl in acetone (20 mg per animal per dose) and failed to find evidence of tumor promotion in 7,12-dimethylbenz(a)anthracene-initiated mice. However, this latter study did not document the impact of the organic solvent (acetone) on the free residual chlorine dose applied to the animals' skin.

It is difficult to compare the results of the present work with these prior studies because of the differing endpoints and method of application. The methodology used in the present study provides a reservoir of active chlorine for some minutes, whereas the doses administered in these prior studies are most likely dissipated within seconds by reaction with organic material on the skin surface. Therefore, although the concentrations applied in the present study are much less than those used in the prior studies, the effective dose might well be higher. It must also be pointed out that these studies addressed the effects of OCl^- only. The present work clearly indicates that HOCl is the more hyperplasiogenic form of aqueous chlorine.

The ability of disinfectant to produce epidermal hyperplasia is of concern because of the excellent correlation between this activity and tumor-promoting ability among phorbol esters (*5,6*). However, this correlation does not hold for compounds of other chemical classes. Since the experiments reported here do indicate an ability of disinfectants to produce epidermal hyperplasia, it is important that they also be evaluated for their tumor-promoting potential in the skin.

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The research described in this article has been reviewed by the Health Effects Research Laboratory and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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